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(54) PROTEINIC COMPOSITIONS

(71) We, ROHM & HAAS G.M.B.H., a German Body Corporate, of Darmstadt, Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to compositions comprising proteinic active compounds bonded to synthetic resin carriers.

It has previously been proposed to bond enzymes to water-insoluble carrier substances whilst still preserving the biological activity of the enzyme and to perform specific degradation reactions with such products. Processes occurring in living tissue are, to some extent, imitated by this type of bonding of biologically active protein derivatives. Thus, in living tissue the protein derivative or a related active substance may be bonded to a cell structure or embedded in membranes, and in this quasi-insoluble form, thereby catalyse biochemical processes. The bonding of the biologically active protein-like substances to a water-insoluble carrier can be adsorptive or covalent. However since a physically adsorbed protein in aqueous suspension is generally in equilibrium with dissolved protein in the aqueous medium it is frequently difficult to avoid an undesired desorption of the protein under conditions encountered in practice. The covalent bonding of the active protein-like substance to a water-insoluble carrier is therefore preferred so that the active substance cannot be readily eluted from the carrier.

Levin, Pecht, Goldstein and Katchalsky have described in "Biochemistry", 3 (12), 1905—1913 (1964), the preparation of a water-insoluble trypsin-synthetic resin composition in which trypsin is coupled to a maleic anhydride-ethylene copolymer cross-linked with hexamethylene-diamine, the unreacted anhydride groups thereof being subsequently hydrolysed. However, the said copolymers are sensitive to hydrolysis, and consequently, during the addition of the typsin to the resin, a number of the anhydride groups are hydrolysed and thereby eliminated as reactive groups for the covalent bonding of the trypsin. Moreover, as our own experiments have shown, a period of about 20 hours may be required for the bonding of the trypsin to the carrier during which period the hydrolysis of the reactive anhydride groups may continue with consequent elimination of these reactive groups. Furthermore the trysin resins obtained must generally be washed for long periods in order to remove by-products such as inactive forms of the resin. The yields of trypsin resin (calculated on the quantity of trypsin used) vary within wide limits (22 to 65%) suggesting that the bonding of the trypsin to the anhydride groups of the abovementioned copolymer is relatively non-specific.

It is an object of the present invention to provide a novel composition comprising a proteinic substance bonded to a synthetic resin carrier.

According to the present invention we provide a water-insoluble composition which comprises a water-insoluble polymer or copolymer of acrylic and/or methacrylic anhydride if desired together with other copolymerisable monomers, said polymer or copolymer containing at least 1% by weight of units derived from the said acrylic and or methacrylic anhydride and if desired being cross-linked, and a proteinic substance (as herein defined) containing at least one α- or ϵ-amino group, the said proteinic substance being covalently bonded to the said polymer or copolymer through reactive groups of the said polymer or copolymer.

The term "proteinic substance" is used herein to include all proteinaceous substances such as proteins per se and protein derivatives and also substances such as enzymes, en-

zyme inhibitors, peptides, polypeptides, antibodies and antigens, and also macromolecular compounds which either contain proteins or are essentially protein-like in nature in that their molecule contains a peptide structure.

In general, the biological activity of the above-mentioned proteinic substance will, to a certain extent, be retained while covalently bonded to the synthetic resin carrier in the compositions according to the present invention.

Examples of enzymes which may be included in the compositions of the invention include trypsin, chymotrypsin, papain, urease, catalase, ribonuclease, streptokinase, diastase and pectinase. Examples of polypeptides which may be included in the compositions of the invention include insulin, oxytocin, vasopressin, hypertensin and adrenocorticotropic hormone (ACTH). As mentioned above antibodies and antigens are also examples of proteinic substances which may be used in accordance with invention. Thus, the compositions of the invention can be used for the specific separation of antibodies and antigens from other natural materials.

Thus, for example, antibodies can be isolated from serum by contacting the serum with a composition according to the invention in which the proteinic substance is the corresponding antigen of the said antibodies in the serum, to form a complex of the antibodies and the "antigen-synthetic resin". This complex may then be removed from the serum, and the antibodies subsequently separated from the "antigen-synthetic resin" in conventional manner, e.g. by elution at an appropriate pH value. The "antigen-synthetic resin" may then if desired, be used for the isolation of further antibodies.

As mentioned above, the polymer or copolymer of acrylic or methacrylic anhydride used in accordance with the present invention is water-insoluble. Thus, water-soluble linear polymers of acrylic or methacrylic anhydride may be cross-linked to form water-insoluble cross-linked polymers of acrylic or methacrylic anhydride which may subsequently be bonded to the proteinic substance. These water-insoluble cross-linked polymers may, in fact, be liable to swell in the presence of water, but in general this does not affect unduly their use in accordance with the invention.

The cross-linking of water-soluble polymers may be effected, for example, by the use of a suitable di- or polyamine or by the use of monomers containing at least two carbon-carbon double bonds in the molecule. The same cross-linking mechanism takes place, as is known, in the reaction of the above-stated homo- or copolymers with other bifunctional compounds, the reactive groups of which react with anhydride groups. Examples of convenient cross-linking agents include ethylene diamine, hexamethylene diamine, propylene diamine, 2,3-diaminopropanol, thioethylamine and polyethylene glycols. It should be noted that the above-mentioned cross-linking of the polymer can also take place simultaneously with the bonding of the polymer to certain of the proteinic substances having two or more groups capable of reacting with the polymer. Cross-linking comonomers which can be used in quantities of about 0.01 to 5% by weight of the total weight of the monomers of the cross-linked copolymer include, for example, vinylacrylate or methacrylate, allyl acrylate or methacrylate, divinylbenzene, ethyleneglycol di-acrylate or dimethacrylate and triallyl cyanurate. In copolymers of acrylic or methacrylic anhydride, cross-linking is not necessary in those cases in which the desired water-insolubility of the polymer is achieved by virtue of the nature and quantity of the comonomer(s) used. The proportion of the polymer constituted by the above-mentioned anhydrides may vary within wide limits. In addition to the acrylic and methacrylic anhydrides, esters, amides and nitriles of acrylic and methacrylic acid, and also of itaconic and crotonic acid may be used as comonomers in the preparation of copolymers for use in the invention. Further examples of such comonomers include styrene and vinyl acetate.

The above-mentioned homo- and copolymers can be prepared as bulk, emulsion, suspension or solution polymers. Dispersion and solution polymers may subsequently be coagulated or precipitated; the precipitation of solution polymers can for example be achieved by subsequent reaction with a cross-linking agent. Dispersions may be coagulated in conventional manner, dried and used in a coarsely dispersed form. In some cases, it may be advantageous to bond the proteinic substance to the synthetic resin particles while in dispersion. The resulting dispersion can then be used, if desired, directly in this form or can subsequently be coagulated, and the coagulate isolated and dried.

According to a further feature of the present invention we provide a process for the preparation of a composition as hereinbefore defined in which an aqueous solution of a proteinic substance containing at least one α- or ϵ-amino group, is contacted with a water-insoluble polymer or copolymer of acrylic and/or methacrylic anhydride if desired together with other copolymerisable monomers containing at least 1% by weight of units derived from the said acrylic and/or methacrylic anhydride and if desired being cross-linked whereby a composition as hereinbefore defined is formed.

The bonding of the proteinic substance to the polymers according to the invention is generally effected by stirring the polymeric carrier with an aqueous solution of the pro-

teinic substance with ice cooling to maintain a temperature between ambient temperature and 60°C; such a temperature does not deleteriously affect the proteinic substance. The composition formed may be subsequently removed from the aqueous medium. As shown in the following examples, a rapid bonding of the proteinic substance to the polymeric carrier occurs in a relatively short time.

The following examples illustrate the invention:—

Example 1

107.4 g. of freshly distilled methacrylic anhydride are mixed with 1 litre of anhydrous benzene and 1.07 g. of benzoyl peroxide are added. The mixture is stirred for 12 hours under a current of nitrogen and at a temperature of 65°C in a flask provided with a reflux condenser and thermometer. The product which precipitates after cooling is filtered with suction, washed with benzene and dried for 10 hours at 45°C. Yield: 107 g. of polymethacrylic anhydride.

5 g. of polymethacrylic anhydride are suspended in a mixture of 150 ml. of water and 10 ml. of methanol. 50 ml. of a 0.5% aqueous solution of hexamethylene diamine are added dropwise with stirring over 10 minutes at room temperature. The reaction mixture is then stirred for a further 15 minutes, and the reaction product filtered off, washed with 50% aqueous methanol and dried in vacuo. Yield: 5.9 g. of cross-linked polymethacrylic anhydride.

3 g. of the cross-linked polymethacrylic anhydride are suspended in a solution of 0.48 g. of crystalline trypsin in 30 ml. of water and stirred for 3 hours at room temperature. The product is then centrifuged and the precipitate washed several times with water and then dried at 0°C in vacuo. Yield: 3.36 g. (96.5% of theory) of "trypsin-resin" containing 13% of protein. The specific activity of the bonded enzyme protein with N-benzoylargenine-p-nitranilide as substrate at pH 8 is 38% of the specific activity of the unbonded crystalline trypsin used.

Example 2

0.6 g. of divinyl benzene and 0.64 g. of benzoyl peroxide are added to 61.6 g. of methacrylic anhydride in 640 ml. of anhydrous benzene and the mixture is polylmerised and worked up as described in Example 1. Yield: 48 g. of cross-linked polymethacrylic anhydride.

1.54 g. of the cross-linked polymethacrylic anhydride are first suspended in 50 ml. of ethanol and partially saponified by the addition of 1.47 g. of 25% aqueous ammonia solution. After 15 minutes the reaction product is filtered off, washed with ethanol and ether and, after drying, suspended in a tenfold excess by weight of a 1.6% aqueous solution of crystalline α-chymotrypsin. The suspension is stirred for 3 hours at room temperature, centrifuged, and the precipitate washed several times with water and dried at 0°C in vacuo. Yield: 0.95 g. of "chymotrypsin-resin" per gram of partially saponified polymethacrylic anhydride (i.e. 82% of theory). The protein content of the "chymotrypsin-resin" is 14%. The specific activity of the bonded enzyme protein with carboxypropionyl-alanine-p-nitranilide as substrate at pH 8 is 75% of the specific activity of the unbonded crystalline α-chymotrypsin used.

Example 3.

150 g. of a mixture consisting of 5% methacrylic anhydride, 30% of methacrylamide, 60% of methacrylic acid and 5% of ethylene glycol dimethacrylate are polymerised in 750 ml. of ethyl acetate with 1.5 g. of azoisobutyric acid dinitrile for 2.5 hours at 75°C in a current of carbon dioxide. The precipitation product is filtered off with suction, washed with ethyl acetate and dried in vacuo. Yield: 145 g. of copolymer.

1.0 g. of the copolymer thus prepared is suspended in 10 ml. of an aqueous solution of 0.18 g. of a pancreas preparation (enzyme content: about 17% trypsin and 11% chymotrypsin) and stirred for 3 hours at room temperature. The suspension is then centrifuged and the precipitate washed several times with water and dried at 0°C in vacuo. Yield: 1.06 g. (90% of theory). The protein content of the product is 7%. The total activity of the enzyme bonded to the resin with N-benzoyl-argenine-p-nitranilide as substrate at pH 8 is 44% of the activity of the enzyme used.

Example 4.

100 g. of a mixture consisting of 10% of methacrylic anhydride and 90% of methyl methacrylate are polymerised in 1 litre of benzene with 1.0 g. of benzoyl peroxide for 12 hours at 65°C and worked up as described in Example 1. Yield: 85 g. of the methyl methacrylate—methacrylic anhydride copolymer.

The water-insoluble copolymer thus prepared can be used for the bonding not only of trypsin but also of other protein-containing active substances.

Example 5.

80 mg. of polymethacrylic anhydride cross-linked with propylene diamine in an analogous manner to that described in Example 1, is suspended in a solution of 40 mg. of crystalline papain in 1 ml. of a 0.05 M phosphate buffer (pH 7.5) and stirred for 4 hours under nitrogen at room temperature. The reaction product is worked up and dried as described in the preceding examples. Yield: 91 mg of "papain resin", i.e. 78% of theory. The protein content of the "papain-resin" is 28%.

The specific activity of the bonded enzyme protein with N-benzoylargenine-p-nitranilide as substrate at pH 8 is 31% of the specific activity of the unbonded crystalline papain used.

WHAT WE CLAIM IS:—

1. A water-soluble composition which comprises a water-insoluble polymer or copolymer of acrylic and/or methacrylic anhydride if desired together with other copolymerisable monomers, said polymer or copolymer containing at least 1% by weight of units derived from the said acrylic and/or methacrylic anhydride and if desired being cross-linked, and a proteinic substance (as herein defined) containing at least one α- or ϵ-amino group, the said proteinic substance being covalently bonded to the said polymer or copolymer through reactive groups of the said polymer or copolymer.

2. A composition as claimed in claim 1 in which the said proteinic substance comprises an enzyme.

3. A composition as claimed in claim 2 in which the enzyme is trypsin, chymotrypsin, papain, urease, catalase, ribonuclease, streptokinase, diastase or pectinase.

4. A composition as claimed in claim 1 in which the said proteinic substance comprises a polypeptide.

5. A composition as claimed in claim 4 in which the polypeptide is insulin, oxytocin, vasopressin, hypertensin or adrenocorticotropic hormone (ACTH).

6. A composition as claimed in claim 1 in which the proteinic substance comprises an antibody.

7. A composition as claimed in claim 1 in which the proteinic substance comprises an antigen.

8. A composition as claimed in any of the preceding claims in which the said copolymer is a copolymer of acrylic and/or methacrylic anhydride and styrene, vinyl acetate and/or at least one ester, amide or nitrile of acrylic acid, methacrylic acid, itaconic acid or crotonic acid.

9. A composition as claimed in any of claims 1 to 7 in which the said polymer is a homopolymer of acrylic or methacrylic anhydride cross-linked with a diamine or polyamine.

10. A composition as claimed in any of claims 1 to 7 in which the said copolymer is a cross-linked copolymer of acrylic and/or methacrylic anhydride and at least one monomer copolymerisable therewith containing at least two carbon-carbon double bonds per molecule.

11. A composition as claimed in claim 10 in which the said copolymerisable monomer comprises vinyl acrylate, vinyl methacrylate, allyl acrylate, allyl methacrylate, divinyl benzene, ethylene glycol diacrylate, ethylene glycol dimethacrylate or triallyl cyanurate.

12. A composition as claimed in claim 10 or claim 11 in which the said copolymerisable monomer is present in an amount of 0.01 to 5% by weight of the total weight of the monomers of the said cross-linked copolymer.

13. A composition as claimed in claim 1 substantially as herein described.

14. A composition comprising a proteinic substance covalently bonded to a synthetic resin substantially as herein described in any of Examples 1—3 and 5.

15. A process for the preparation of a composition as claimed in claim 1, in which an aqueous solution of a proteinic substance containing at least one α- or ϵ-amino group, is contacted with a water-insoluble polymer or copolymer of acrylic and/or methacrylic anhydride if desired together with other copolymerisable monomers containing at least 1% by weight of units derived from the said acrylic and/or methacrylic anhydride and if desired being cross-linked whereby a composition as claimed in claim 1 is formed.

16. A process as claimed in claim 15 in which the proteinic substance is contacted with the said water-insoluble polymer at a temperature between ambient temperature and 60°C.

17. A process as claimed in claim 15 or claim 16 in which the water-insoluble polymer is employed in the form of a dispersion.

18. A process as claimed in claim 17 in which the resulting composition is obtained in the form of a dispersion in the aqueous solution, the said dispersion then being subsequently coagulated and isolated from the aqueous solution.

19. A process as claimed in claim 15 substantially as herein described.

20. A process for the preparation of a composition comprising a proteinic substance covalently bonded to a synthetic resin substantially as herein described in any of Examples 1—3 and 5.

21. A composition as claimed in claim 1 whenever prepared by a process as claimed in any of claims 15 to 20.

22. A dispersion or suspension of a composition as claimed in any of claims 1 to 14 and 21 in an aqueous medium.

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